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Treatment of Memory Impairment and Sensorimotor Deficits in an Animal Model for the Gulf War Veterans' Illnesses

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Table of Contents

Introduction

The main goal of this project is to investigate the use of flupirtine to protect and/or treat the Gulf War Veterans' Illnesses (GWVI) in an animal model. Many of the Persian Gulf War (PGW) veterans have complained of illnesses, known as the GWI affecting the nervous and the musculoskeletal systems (Institute of Medicine 1995). The symptoms include chronic fatigue, muscle pain, forgetfulness, and inability to concentrate. During the war, American military personnel were exposed to a combination of chemicals such as the insect repellent DEET and the insecticide permethrin to protect against insect borne diseases. Sub-chronic daily dermal exposure of rats, for 60 days, to 40 mg/kg DEET and 0.13 mg/kg permethrin; doses comparable to those present during the PGW environment, resulted in the development of a rat model of the GWI that produced neurobehavioral consequences consistent with those reported by the Veterans(Abou-Donia et al., 2001, 2004). Neuropathological alterations were diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in the cerebellum (Abdel-Rahman et. al., 2001). These treatments also resulted in sensorimotor deficits (Abou-Donia, et al., 2004).

Several studies have demonstrated that combined dermal exposure to both DEET and permethrin resulted in biomarkers of oxidative stress and apoptosis: induction of 8-hydroxy-2'-deoxyguanosine (Abu-Qare and Abou-Donia, 2000), release of cytochrome c (Abu-Qare and Abou-Donia, 2001), increased urinary excretion of 3-nitr0tyrosine (Abu-Qare et al., 2001). The results suggest that neuronal regional brain cell death is caused by the production of reactive oxygen species induced by combined exposure to DEET and permethrin, and subsequent apoptotic cell death. Typically, equilibrium exists between generation of ROS and the antioxidant defense system, such as glutathione peroxidase, glutathione reductase, cacalase, superoxide dismutase, and cellular thiols such as glutathione.

Flupirtine has been used in the treatment of patients with memory and sensorimotor deficits. Oral daily doses of flupirtine had beneficial effects on patients with Creutzfeldt-Jacob disease (Otto et al., 2004) and of neuronal damage following global ischemia in rats (Block et al., 1997). This study also showed that neuronal damage in CA1 of the hippocampus was significantly reduced. Flupirtine greatly reduced neurotoxicity caused by a prion protein fragment (Perovic et al., 1997). An early study by Riethmuller-Winzen (1987) found that flupirtine to be safe and efficacious in several randomized, controlled, double-blind clinical trials for patients. Furthermore, flupirtine was effective in treating headache/migraine and abdominal spasms (Friedel and Fitton, 1993). These studies indicated that treatment with flupirtine resulted in improvement of the patients' quality of life. Also, the findings that flupirtine does not induce development of dependence or tolerance and does not have prominent side effects (Friedel and Fitton, 1993), make it a promising and clinically safe drug for treatment of veterans with GWI.

Body

Hypothesis. We hypothesized that flupirtine, a non-opiate analgesic that has been used in the treatment of memory deficits and of muscular diseases in patients, could treat the Persian Gulf War Veterans, whose major complaints include memory impairment and sensorimotor deficits.

Specific Aims. The specific aims are carried out following combined dermal exposure of adult rats to 40 mg/kg DEET and 0.13 mg/kg permethrin, alone or in combination with flupirtine at 5 or 10 mg/kg, in two experimental paradigms:

1) Concurrent exposure to DEET/permethrin and flupirtine for 60 days (year 1), and
2) Exposure to DEET/permethrin for 60 days, then after 24 hours followed by flupirtine for an additional 60 days (year 2).
Animals are evaluated for neurological deficits by determining: clinical signs, sensorimotor and cognition functions, oxidative stress, apoptosis, and neuronal and glial cell morphology.

Methods
Test chemicals

DEET (97.7%), *N,N*-diethyl *m*-toluamide) was purchased from Sigma Chemical Company (ST. Louis. MO). Technical grade permethrin, (-+)-cis/trans-3-(2,2-dichloroethynyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester (93.6%), was obtained from Roussel Uelaf Corporation (Pasadena, TX). Flupirtine maleate (98%) was purchased from Tocris Bioscience (Ellisville, Mo).

Animals
Adult male Sprague-Dawley rats weighing approximately 225 g were obtained from Zivic-Miller Laboratories (Allison Park, PA). The animals were randomly assigned to bcontrol and treatment groups and housed at 21-23 ºC with a 12-h light/dark cycle. The animals were supplied with feed (Purina certified rodent chow, Ralston Purina, St. Louis, MO) and tap water *ad libitum*. The rats were allowed to adjust to their environment foe a week before starting the treatment. The protocol for the treatment was approved by the DOD and Duke University Institutional Animal Care and Use Committees (IACUC).
Treatment protocol
Protection from DEET/permethrin-induced neurotoxicity with flupirtine:
Four groups (n = 20) of rats received were treted as follows:

a. Control (vehicle), daily for 60 days,
b. DEET/permethrin, daily for 60 days,
c. Flupirtine in water (1ml/kg) daily for 60 days,
d. DEET/permethrin + 10 mg/kg flupirtine in water (1 ml/kg) daily for 60 days.

Assays:
1. The animals were weighed and their clinical condition was monitored daily.
2. Twenty four hours after the 60-day treatment period, the animals were evaluated for neurobehavioral , biochemical, and pathological parameters:
   a. A group of 10 rats from each group will be evaluated for cognition using Morris Water Maze test and the other group of 10 animals will be tested for sensorimotor performance.
   b. Neuropathological alterations were carried out in 10 rats; 5 from each behavioral sub-group.
   c. Biochemical assays for the oxidative stress were carried out in 10 animals; 5 from each behavioral sub-group.

Behavioral Evaluation

A group of 10 rats were evaluated for cognition using Morris Water Maze, and another group of 10 animals will be tested for sensorimotor performance.

1. **Learning and Memory**

Spatial learning and memory were assessed using the Morris Water Maze (MWM, Morris, 1981; D'Hooge and De Deyn, 2001). This task requires animals to use distal visual cues in learning the location of a platform hidden below the surface of the water. Spatial memory acquisition in the MWM is known to be sensitive to dysfunction of hippocampus, though dysfunction within striatum, basal forebrain, cerebellum, and neocortex can adversely affect other aspects of MWM performance (D'Hooge and De Deyn, 2001).

Brief overview of methods: Animals were randomized to one of four treatment groups: DEET/Perm + flupirtine, DEET/Perm + vehicle, vehicle + flupirtine, or vehicle + vehicle. They were dosed accordingly for 60 days and then run in the water maze beginning day 61.

1. The water maze task consisted of 6 daily swim trials starting from 3 different starting points for 4 consecutive days. All trials were terminated at 60s or when the animal climbed onto the hidden platform. At the end of each trial animals were placed on the platform for 15s and then given 30s respite between trials. Day 5 consisted of a 60s probe trial in the absence of the hidden platform.
2. Learning is established by a decline in mean distance swum across days. The dependent measure was the distance swum from the starting point to the end of the trial. All swim paths were visually inspected for tracking artifacts. Where artifacts were observed, trials were either retracked using the original video file or they were eliminated from the analysis. The mean distance swum for all usable trials was calculated and used as the primary dependent measure.
3. Probe trial performance was assessed using the mean distance from the former goal location (proximity). Probe trial performance can be complicated because some animals will swim directly to the former goal location and then either continue to search in that region or begin to search in other areas of the tank. Conversely, some animals may have poor recall for the platform location and swim farther before reaching that location. To address this heterogeneity in performance, the 60s probe trial was divided into 4 epochs of 15s.

2. Sensorimotor Assessments

These behavioral tests measure sensory and motor reflexes, motor strength, spontaneous locomotors activity, and coordination. The results of these test are being analyzed.

Histopathological procedure

Each animal was perfused transcardially, first with 100 ml of normal saline containing 0.1% heparin and then with 450 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) over a period of 30 minutes. (Abdel-Rahman *et al*, 2001; Abou-Donia *et al,* 2003).

Results

Clinical Condition

All animals survived the experimental period, and no visual differences were noted in general condition between control and treatment groups. Body weight was not significantly different between treatment and control groups.

Standard learning analyses:
We employed a 2 (DEET/perm vs vehicle) x 2 (flupirtine vs. vehicle) x 4 (day) repeated measure ANOVA (RM-ANOVA) with Day as the repeated measure. Fig 1 displays the mean daily swim distance for each group. Analysis reveals:

1. A significant effect of Day, $F(3,108) = 57.58$, $p < .009$ and an interaction between Day and DEET/perm, $F(3,108) = 3.23$, $p = .025$. There were no other main effects or interactions.
2. The main effect of Day indicates that all groups learned the platform location while the interaction reveals a subtle difference in the rate of learning achieved by DEET/perm treated animals relative to vehicle treated animals.
3. Visual inspection of figure 1 and corresponding follow-up tests reveal that DEET/perm treated animals swam further on Day 1 than did vehicle treated animals ($p < .05$).
4. The reverse occurred on Day 2 with DEET/perm animals performing better than vehicle treated animals **(**$p < .05$). No such differences occurred on Day 3 or 4.

Probe Trial Analyses:
We employed a similar 2 x 2 x 4 RM-ANOVA as described above. However, this time the repeated measure was the four time epochs created for the 60s probe trial described above: 0-15s, 15-30s, 30-45s, and 45-60s.

1. Initial analyses reveal a significant effect of Epoch, $F(3,108) = 5.6$, $p=.001$. This reflects a general trend toward an animal's initial search for the platform close to its former location and then expanding it's search in more distant aspects of the field later in the 60s trial (as can be seen in figure 2).
2. There are no other significant main effects or interactions except for the 3-way interaction between Epoch, DEET/perm, and Flupritine, $F(3,108)=3.03$, $p=.03$.
3. To better understand this interaction we completed a series of simple interaction analyses. The first set of analyses investigated the effects of DEET/perm and Flupirtine at each individual time point. This analysis revealed no significant main effects or interactions. From this we concluded that change across epochs was essential in understanding the 3-way interaction.
4. As a result, we performed a 2 (flupirtine vs. vechicle) x 4 (epochs) RM-ANOVA for animals that received vehicle and then again for animals that received DEET/Perm (see figures 2a and 2b). For vehicle treated animals, analysis revealed a significant effect of Epoch, $F(3,54) = 3.2$, $p=.03$, no effect of flupirtine and no interaction indicating that animals swam further from the former platform location as time passed (independent of whether they received flupirtine or vehicle - figure 2a).
5. For DEET/Perm treated animals, there was a significant effect of Epoch, $F(3,54) = 3.72$, $p=.02$, a significant interaction between Epoch and Flupirtine**,** $F(3,54) = 3.7$, $p=.02$, and no main effect of Flupirtine (figure 2b).
6. Simple-simple main effect analyses revealed that animals treated with DEET/Perm + vehicle showed no increased exploratory behavior beyond the former platform location ($p=.92$).
7. However, animals treated with DEET/Perm + flupirtine showed significant increased exploratory behavior beyond the former platform location as time progressed, $F(3,24) = 5.8$, $p=.004$.

Preliminary Histological Analysis

A few representative perfused-fixed brains from each of the treatment groups were examined by coronal and/or parasagittal sectioning through the entire brain. H&E and Nissl staining was done at multiple levels through each brain, and microscopic examination was done at all of these levels to look for histological abnormalities (differences in overall architecture, cell number, morphology, and staining characteristics) between three treated groups (Flupirtine/DEET/Permethrin; DEET/Permethrin; Flupirtine only) and control (vehicle).

The main changes relative to control were seen in the cerebellum:

(1) Increased numbers of cells in the Purkinje layer with increased staining intensity. There were small numbers of these more densely-stained cells in the control cerebellum, but there were many more of them in all three experimental groups. These cells were not dead, as they retained their nuclear profile, but their cellular features were less distinct and their cytoplasm seemed more condensed.

(2) Increased numbers of cells in the granular cell layer with angulated nuclei that were smaller and darker than the granule cells. There were small numbers of these cells in the control cerebellum, but they were markedly increased in number in all three experimental groups. These cells may represent granule cells or some other interneuron subtype that have suffered damage, or they may be microglia or some other inflammatory cell type.

(3) A less consistent change was the appearance in the Purkinje layer of cells with small nuclei and perinuclear clearing, perhaps representing a glial reaction.

In some areas, the changes were seen together (as in the microphotograph), but they were also seen separately in other areas. There did not seem to be a specific location in the cerebellum to which these changes were restricted.

No significant abnormalities were seen relative to controls in the cerebral cortex, hippocampus, or other major regions of the forebrain, midbrain or hindbrain. The sub-ventricular zone, rostral migratory stream, and olfactory bulbs showed no significant difference between control and experimental brains, so there does not seem to be an obvious effect on this major site of neurogenesis.

Only one or two brains have has been examined in detail from each of the four groups so far, so these findings are very preliminary. It is not yet possible to compare the relative extent of changes between the different experimental groups. We will expand our initial analysis to include several of each group, and will use adjacent unstained sections to conduct an immunohistochemical characterization of the affected cell types, and assess their viability and mitotic activity.

Key Research Accomplishments

The main goal of this project is to investigate the protection and/or treatment of the Gulf War Veterans' Illnesses (GWVI) in an animal model that was developed for this condition. We have been studying the ability of flupirtine to protect and/or treat from GWVI in a rat model that we established for GWVI. Flupirtine is an approved medication for human use in several diseases such as Alzheimer's disease to treat memory impairment. This report represents the results of the first year of the project that was designed to investigate the use if flupirtine to protect from GWVI in our rat model. There was a delay before starting animal studies in order to coordinate approvals of the animal protocol among DOD, Duke University and the VA where the animals were kept to

allow Water Maze testing without moving the animals back and forth from another site for testing. As a result, we present here preliminary data, while the rest of the results are being analyzed.

Reportable outcomes

The results of the first year confirmed previous findings that our rat model for the GW VI was reproduced using daily treatment with dermal doses of DEET and permethrin for 60 day. Although, these animals were not clinically different from controls, they developed alterations in memory and neuropathology.

Conclusion

The preliminary results of the first year confirmed our previous reports that treatment rats with daily dermal doses of DEET and permethrin produces an animal model for GW VI. The complete picture will be developed once we finish all of the results.

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Appendices

None